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Comparative Evaluation of Fractionated *Piper crocatum* Ruiz & Pav. Leaf Extracts on Wound Healing in a Mice Excision Model

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Abstract: Wound healing is a dynamic physiological process influenced by various cellular responses and biochemical mediators. This study aimed to evaluate the wound-healing activity of fractionated extracts of *Piper crocatum* using a mouse excision wound model and to determine the phytochemical constituents associated with its therapeutic effects. Methanolic leaf extracts were fractionated into *n*-hexane, ethyl acetate, and residual fractions, followed by quantification of total phenolic and flavonoid contents and topical application for 14 days. Wound area, contraction percentage, and histological changes were assessed to compare treatment outcomes among groups. The residual fraction exhibited the highest phenolic and flavonoid contents (TPC = 50.85 ± 1.65 mg GAE/g and 15.36 ± 2.07 mg QE/g, respectively), whereas the ethyl acetate fraction contained moderate phenolic levels (49.81 ± 1.33 mg GAE/g) without detectable flavonoids. Both fractions significantly enhanced wound contraction compared with the negative control and achieved near-complete closure by Day 14, accompanied by improved fibroblast proliferation and collagen organization. The findings demonstrate fraction-dependent differences in wound-healing efficacy and suggest a potential association between phenolic-rich fractions and enhanced tissue repair. While mechanistic pathways were not directly investigated, these results provide a foundation for further molecular and pharmacological studies to clarify the bioactive constituents and their roles in wound regeneration.

Keywords: *Piper crocatum*; wound healing activity; phenolic content; flavonoid content; excision wound model.

1. Introduction

Wound healing is a natural process involving phases of hemostasis, inflammation, proliferation, and maturation [1]. Each phase contributes to tissue restoration: hemostasis forms clots, inflammation clears debris, proliferation promotes cell growth and collagen production, and remodeling refines tissue structure. Enhancing this process with agents that accelerate healing and collagen synthesis can reduce discomfort and prevent complications [2]. Disruptions in any of these phases may impair tissue repair, slow re-epithelialization, trigger excessive inflammation, or promote microbial colonization, ultimately increasing the risk of

delayed healing, scarring, or chronic, non-healing wounds [3]. These challenges underscore the urgent need for wound therapies that are not only effective but also safe, affordable, and widely accessible [4].

In recent decades, natural products have gained increasing scientific attention as potential wound-healing agents due to their abundance, structural diversity, and broad spectrum of biological activities [5]. Plant-derived metabolites, including phenolics, flavonoids, tannins, alkaloids, and terpenoids, play important roles in modulating key pathways across multiple phases of the wound-healing cascade. Their antioxidant properties mitigate oxidative stress at the injury site, anti-inflammatory activities regulate the inflammatory response, and antimicrobial effects minimize the risk of infection [6]. Furthermore, several phytochemicals have been shown to stimulate fibroblast proliferation, angiogenesis, and extracellular matrix (ECM) synthesis, thereby accelerating collagen deposition and tissue remodeling [7].

Among traditionally used medicinal plants, *Piper crocatum* Ruiz & Pav., locally known in Indonesia as red betel (sirih merah), has been widely employed for treating skin injuries, ulcers, and inflammatory conditions [8-10]. Phytochemical investigations have revealed the presence of phenolic compounds, flavonoids, glycosides, tannins, saponins, alkaloids, and chromene derivatives such as pipercrosides A and B [9,11]. Experimental studies have suggested that *P. crocatum* extracts may influence wound-related pathways, including modulation of p53, E-cadherin, and SOD1 expression in hyperglycemic fibroblast models, as well as regulation of IL-10, TGF-β1, VEGF expression, and collagen density in excision wound models [9,12]. These findings collectively indicate the biological relevance of this plant in tissue repair processes.

Despite these promising reports, several limitations remain in the existing literature. Most prior investigations have relied on crude extracts, which contain complex mixtures of phytochemicals with varying polarity and bioactivity. Such approaches limit the ability to determine whether wound-healing effects are uniformly distributed across constituents or concentrated within specific solvent fractions. Moreover, although certain molecular markers have been examined in selected models, systematic comparative evaluation of solvent-partitioned fractions in a standardized *in vivo* excision wound model remains limited. Consequently, the relationship between fraction-dependent phytochemical composition and functional wound-healing efficacy has not been comprehensively established.

To address these gaps, the present study aimed to compare the in vivo efficacy of fractionated methanolic extracts of *P. crocatum* leaves using a murine excision wound model. The crude extract was fractionated into *n*-hexane, ethyl acetate, and residual fractions to evaluate solvent-dependent variations in phytochemical composition. Total phenolic and flavonoid contents were quantified, and wound contraction as well as histological parameters were evaluated to examine potential associations between phytochemical content and tissue repair outcomes. By integrating phytochemical profiling with macroscopic and histological assessments, this study aims to provide a clearer, more systematic understanding of fraction-dependent wound-healing activity while avoiding premature mechanistic conclusions. These findings are intended to serve as a foundation for future molecular investigations and further pharmacological characterization.

2. Materials and Methods

2.1. Plant material.

Leaf samples were collected from Muaro Bulian City, Jambi, Indonesia. Botanical identification was conducted at the Plant Taxonomy Laboratory, Department of Biology, FMIPA Universitas Padjadjaran, and verified under identification letter No. 35/HB/04/2024, confirming the species as *P. crocatum* Ruiz & Pav. The collected leaves were dried in an oven at 55°C, yielding 20.64% (w/w) of dried plant material.

2.2. Instruments.

The instruments employed in this study included a UV–Vis spectrophotometer (Thermo Scientific GENESYS®), a hot plate stirrer (VELP Scientifica®), an oven (Memmert®), a water bath (Memmert®), an analytical balance (Kern®), a rotary evaporator (IKA® RV10), and a microtome (Leica® RM2125).

2.3. Research material and reagents.

The materials used in this study included methanol (Brataco®), n-hexane (Brataco®), ethyl acetate (Brataco®), acepromazine maleate (Castran®), gallic acid (Sigma–Aldrich®), quercetin (Sigma–Aldrich®), Folin–Ciocalteu reagent (Sigma–Aldrich®), sodium acetate (Merck®), Vaselinum flavum (Brataco®), distilled water (Ikapharmindo®), depilatory cream (Veet®), povidone–iodine ointment (Betadine®), animal feed, formaldehyde solution (Formalin®), ethanol (Brataco®), xylene (Merck®), and paraffin (Leica®).

2.4. Extraction and fractionation procedure.

The extraction was performed using cold maceration. A total of 500 g of powdered P. crocatum leaves was immersed in 70% methanol (1:10 w/v) for 72 h at room temperature with intermittent stirring. The mixture was filtered, and the residue was re-macerated twice under identical conditions to ensure exhaustive extraction. All filtrates were combined and concentrated under reduced pressure at 40°C using a rotary evaporator to obtain the crude methanolic extract. The yield of dried simplicia obtained from fresh leaves was 20.64% (w/w). The yield of the crude methanolic extract relative to dried simplicia was 17.14% (w/w), which complies with the minimum extractive value requirement specified in the Indonesian Herbal Pharmacopeia (≥ 17%). Fractionation was carried out using liquid–liquid partitioning based on solvent polarity. The crude extract was suspended in distilled water and sequentially partitioned with n-hexane and ethyl acetate (1:1 v/v each) in a separatory funnel. After each partition, the organic layer was collected separately, and the remaining aqueous phase was retained as the residual fraction. All fractions were evaporated to dryness under reduced pressure and stored in amber vials at 4°C until further analysis [13].

2.5. Determination of total phenolic content.

The determination of total phenolic content was carried out following the procedure described by Efendi *et al*. with slight modifications [14]. An aliquot of the extract (0.2 g) was dissolved in methanol *p.a*. and diluted to 25 mL after magnetic stirring and filtration. Gallic acid (10 mg/25 mL) was prepared as the reference standard and serially diluted to obtain 5–

100 µg/mL calibration solutions. For analysis, 1 mL of the sample or standard solution was mixed with 5 mL of Folin–Ciocalteu reagent (7.5%). After 8 min, 4 mL of 1% NaOH was added, and the mixture was incubated for 1 h at room temperature. Absorbance was measured at ~730 nm against a reagent blank. TPC was calculated from the gallic acid calibration curve and expressed as mg gallic acid equivalents (GAE) per gram of extract. All measurements were performed in triplicate. Calibration curves were constructed from standard solutions, and linear regression was used for quantification.

2.6. Determination of total flavonoid content.

The determination of total flavonoid content was carried out following the method described by Efendi *et al.* [14]. A total of 0.2 g extract was dissolved in methanol *p.a.*, stirred for 30 min, filtered, and diluted to 25 mL. Quercetin (10 mg/25 mL) was prepared as the reference standard and diluted to obtain 25–100 µg/mL calibration solutions. For analysis, 0.5 mL of the sample or standard was mixed with 1.5 mL methanol *p.a.*, 0.1 mL of 10% $AlCl_3$, 0.1 mL sodium acetate (1 M), and 2.8 mL distilled water. The mixture was incubated for 30 min at room temperature, and absorbance was measured at 425 nm against a blank (prepared without $AlCl_3$). Total flavonoid content was calculated from the quercetin calibration curve and expressed as mg quercetin equivalent (QE) per gram of extract. All measurements were performed in triplicate. Calibration curves were constructed from standard solutions, and linear regression was used for quantification.

2.7. Wound healing activity.

All animal procedures complied with institutional guidelines and were approved by the Research Ethics Committee of the Faculty of Medicine and Health Sciences, Universitas Jambi (Approval No. 986/UN21.8/PT.01.04/2024). Male mice (20–30 g) were acclimatized for 7 days prior to experimentation. The animals were randomly assigned into five groups (n = 5 per group) using a simple random allocation method: negative control (Vaselinum flavum), positive control (povidone–iodine ointment 10%), and three treatment groups receiving 10% *n*-hexane, ethyl acetate, or aqueous residual fractions of *P. crocatum*. The 10% concentration was selected as it reflects a commonly used concentration in traditional herbal infusion preparations and was applied uniformly across all fractions to enable comparative evaluation. The dorsal area was shaved, depilated using a commercial depilatory cream, and disinfected with ethanol. Under anesthesia induced by acepromazine maleate, a standardized full-thickness excision wound (~2 cm in diameter) was created using sterile surgical scissors. Test formulations were applied topically twice daily for 14 days. Wound area analysis was performed using digital image analysis (ImageJ). Wound progression was evaluated on days 3, 7, and 14 to determine wound area, and the percentage of wound closure was calculated using the formula:

$$P\ (\%) = \frac{d_1 - d_x}{d_1}\ x\ 100 \qquad (1)$$

Where d_1 represents the initial wound area, and d_x represents the wound area on the respective evaluation day. For histological assessment, wound tissue biopsies were collected on days 3, 7, and 14, fixed in 10% formalin, processed using a standard paraffin-embedding protocol, sectioned at 3–5 µm, and stained with hematoxylin–eosin. The histological slides were evaluated by an independent observer blinded to the treatment groups. Microscopic

evaluation focused on collagen fiber density, epithelial regeneration, and restoration of dermal tissue architecture [15].

2.8. Data analysis.

Data are expressed as mean ± standard deviation (SD). Normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test) were assessed prior to ANOVA. One-way ANOVA was used for phytochemical analysis, while two-way ANOVA was applied for wound contraction analysis across time and treatment. Post hoc comparisons were performed using Duncan's Multiple Range Test, with $p < 0.05$ considered statistically significant.

3. Results and Discussion

3.1. Total phenolic content (TPC) and total flavonoid content (TFC).

The phytochemical quantification revealed a clear fraction-dependent distribution of phenolic and flavonoid constituents in the methanolic extract of *P. crocatum* (Table 1). The residual fraction exhibited the highest TPC = 50.85 ± 1.65 mg GAE/g, closely followed by the ethyl acetate fraction (TPC = 49.81 ± 1.33 mg GAE/g). In contrast, the *n*-hexane fraction contained markedly lower phenolic levels (TPC = 31.41 ± 2.13 mg GAE/g). TFC was detected exclusively in the residual fraction (15.36 ± 2.07 mg QE/g), whereas no measurable flavonoids were observed in either the *n*-hexane or ethyl acetate fractions. Consequently, the residual fraction of *P. crocatum* may contain a higher proportion of hydrophilic phenolic and flavonoid constituents, consistent with solvent polarity characteristics. This distribution pattern aligns with the known solubility characteristics of phytochemicals: phenolic acids and simple polyphenols typically partition into solvents of moderate to high polarity, whereas flavonoid glycosides, owing to their hydrophilic nature, tend to remain concentrated in the most polar or aqueous residual fractions [16].

Table 1. Total phenol and total flavonoid content of *Piper crocatum* fraction.

Fraction	TPC (mg GAE /g)	TFC (mg QE/ g)
n-hexane	31.41 ± 2.13^{a}	-
ethyl acetate	49.81 ± 1.33^{b}	-
Residual	50.85 ± 1.65^{c}	15.36 ± 2.07

Values are expressed as mean ± SD. Different superscript letters within each column indicate statistically significant differences ($p < 0.05$).

Polyphenols are widely recognized for their strong antioxidant capacity, free-radical–scavenging activity, and their ability to modulate both inflammatory and proliferative pathways, all of which play critical roles in wound-healing mechanisms [17,18]. However, oxidative stress markers and inflammatory mediators were not directly quantified in the present study; therefore, the biological relevance of phenolic content should be interpreted cautiously. Catechaldehyde identified in *P. crocatum* has also been reported to exhibit anti-inflammatory potential [11]. Consistent with this, a recent review identified flavonoids as "potential wound-healing molecules" due to their anti-inflammatory, antioxidant, pro-angiogenic, and re-epithelialization effects [3]. Additionally, phenolic constituents from medicinal plants have consistently been associated with enhanced tissue repair through combined antioxidant protection, antimicrobial activity, and stimulation of collagen synthesis [19].

3.2. Evaluation of wound healing activity.

Wound area progressively decreased in all experimental groups throughout the 14-day observation period. Baseline wound sizes on day 0 were comparable among groups, indicating uniform initial conditions. By Day 14, the positive control and the residual fraction achieved complete wound closure, whereas the ethyl acetate fraction exhibited near-complete closure with only minimal residual area. In contrast, the *n*-hexane fraction and the negative control showed slower wound contraction and larger residual wound areas (Table 2 and 3, and Figure 1).

Statistical analysis supported these observations. Two-way ANOVA revealed significant effects of treatment, time, and their interaction on wound area ($p < 0.05$). Duncan's post hoc test confirmed that the negative control differed significantly from all treated groups, indicating clear wound-healing benefits of the *P. crocatum* fractions. No significant differences were observed among the positive control, residual, and ethyl acetate fractions, suggesting that these fractions demonstrated healing performance comparable to that of the standard treatment under the experimental conditions. In contrast, the *n*-hexane fraction was significantly less effective than other fractions.

Table 2. Wound area of groups after treatment.

Treatment groups	Wound area on days (cm^2)					Mean ± SD
	0	3	7	10	14	
positive control	3.42 ± 0.39	2.59 ± 0.30	1.66 ± 0.17	0.95 ± 0.11	0.00 ± 0.00	1.73 ± 1.24^{a}
negative control	3.55 ± 0.55	2.85 ± 0.46	2.34 ± 0.38	1.90 ± 0.32	1.31 ± 0.22	2.34 ± 0.87^{c}
n-hexane fraction	3.25 ± 0.92	2.55 ± 0.71	1.92 ± 0.56	1.43 ± 0.41	0.75 ± 0.20	2.02 ± 1.04^{b}
ethyl acetate fraction	3.13 ± 0.35	2.43 ± 0.28	1.63 ± 0.18	1.01 ± 0.10	0.15 ± 0.04	1.66 ± 1.08^{a}
residual fraction	3.51 ± 0.62	2.69 ± 0.49	1.74 ± 0.29	1.01 ± 0.17	0.00 ± 0.00	1.75 ± 1.30^{a}
Mean ± SD	3.30 ± 0.57^{t}	2.66 ± 0.45^{s}	1.86 ± 0.41^{r}	1.26 ± 0.44^{q}	0.44 ± 0.54^{p}	

Values are expressed as mean ± SD. Different superscript letters within the same column indicate inter-group differences at the same time point, while different superscript letters within the same row indicate intra-group differences across time ($p < 0.05$).

Macroscopic observations (Table 3) were consistent with these findings. The negative control showed delayed scab formation, persistent inflammation, and incomplete epithelialization at Day 14. In contrast, ethyl acetate and residual fractions promoted rapid granulation tissue development, early scab maturation, and near-complete re-epithelialization, closely resembling the healing profile observed in the positive control. Although *n*-hexane produced noticeable improvement compared with the negative control, it did not achieve full wound closure.

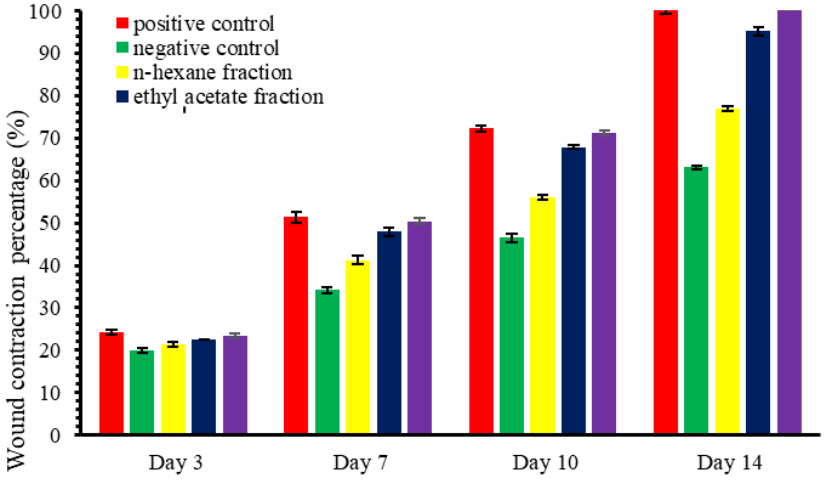
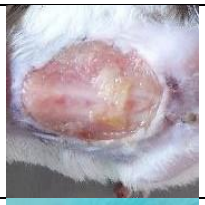
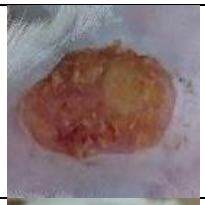
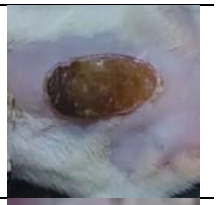
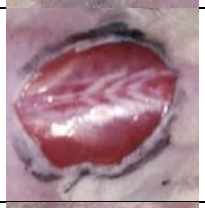
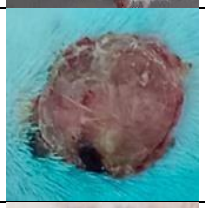
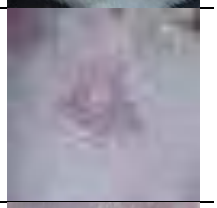
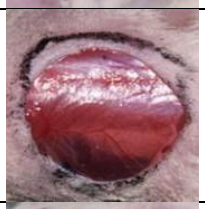
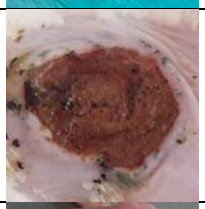
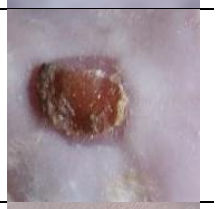
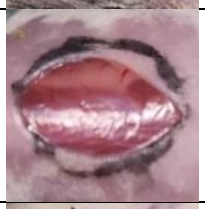
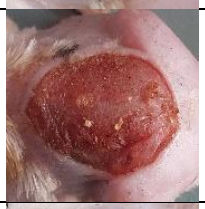
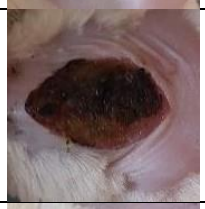
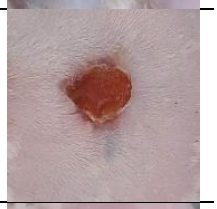
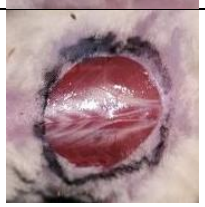
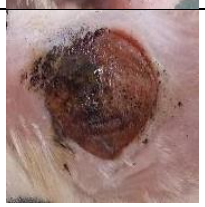
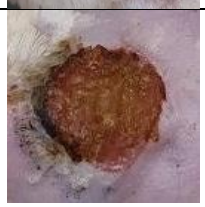


Figure 1. Percentage of wound contraction following treatment with *P. crocatum* fractions.

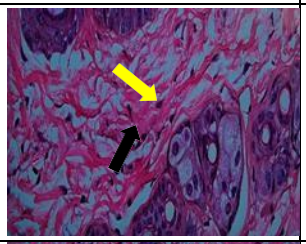
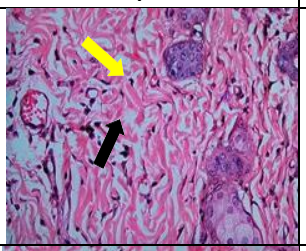
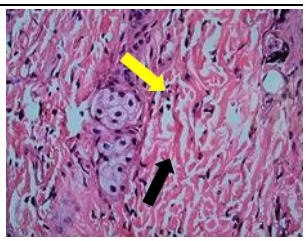
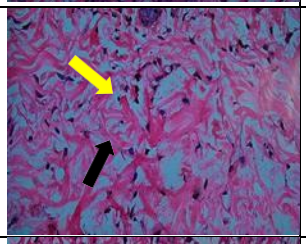
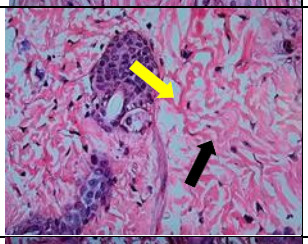
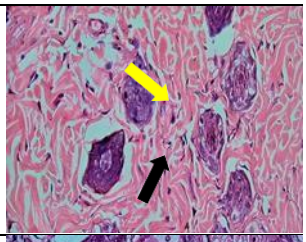
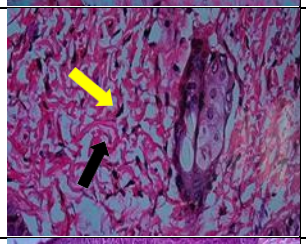
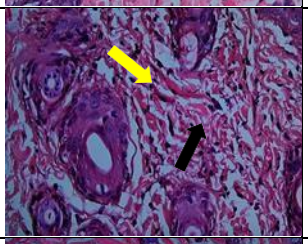
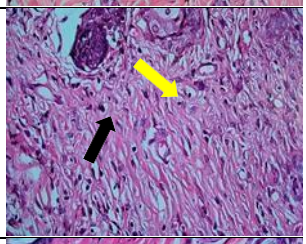
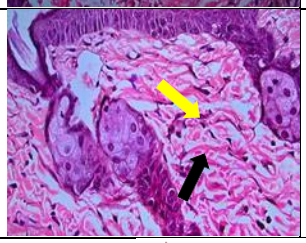
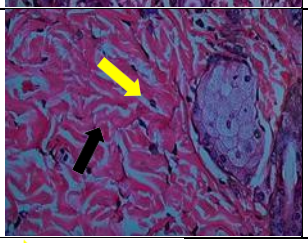
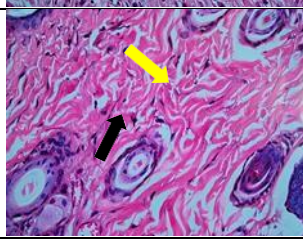
Table 3. Wound contraction profiles of each treatment group over 14 days of observation.

Treatment groups	Days			
	0	3	7	14
negative control				
positive control				
n-hexane fraction				
ethyl acetate fraction				
residual fraction				

Histological analysis further substantiated the superior efficacy of ethyl acetate and residual fractions (Table 4). On Days 7 and 14, both fractions exhibited increased fibroblast proliferation and more organized collagen deposition compared with the negative control. These parameters are commonly associated with progression from the proliferative to the remodeling phase of wound repair [9]. The enhanced fibroblast density and collagen maturation in ethyl acetate and residual fractions align with their greater wound contraction and higher percent closure. Similar findings have been reported in other Piper species, where treatment increased fibroblast proliferation and collagen organization by modulating E-cadherin and SOD1 expression [20]. It should be noted that histological evaluation was qualitative in nature, and no quantitative histomorphometric scoring or biochemical collagen quantification was performed in this study.

Phenolic and flavonoid compounds are recognized for their antioxidant, anti-inflammatory, and antimicrobial properties, all of which support the wound-healing process by modulating inflammation, reducing oxidative stress, stimulating fibroblast proliferation, and improving collagen organization [3,21]. The superior healing observed in the ethyl acetate and residual fractions may be associated with their higher phenolic concentrations. Furthermore, the presence of flavonoids exclusively in the residual fraction may have contributed to its slightly improved healing performance compared with the ethyl acetate fraction, as flavonoids are known to promote angiogenesis, extracellular matrix synthesis, and epithelial regeneration [12,22].

Table 4. Histological examination of wound healing following treatment application.

Treatment groups	Days		
	3	7	14
Negative control			
n-hexane fraction			
ethyl acetate fraction			
residual fraction			

collagen; = fibroblast

Taken together, the results suggest that the ethyl acetate and residual fractions exhibit enhanced wound-healing activity under the present experimental conditions, with efficacy comparable to that of the standard treatment. The observed activity of these fractions appears to correlate with their phytochemical composition; however, direct causal relationships cannot be established from the present data. These findings identify the ethyl acetate and residual fractions as candidates for further phytochemical characterization and mechanistic investigation. Future studies should incorporate molecular analyses such as VEGF and TGF-β1 expression, hydroxyproline quantification, and evaluations in impaired wound models to clarify their mechanisms of action and therapeutic relevance [23,24].

4. Conclusions

This study demonstrates fraction-dependent differences in the wound-healing activity of *P. crocatum* leaf extracts in a murine excision wound model. Among the tested fractions, the ethyl acetate and residual fractions exhibited more pronounced wound contraction and improved histological features compared with the *n*-hexane fraction and the negative control. By Day 14, the residual fraction had achieved complete wound closure, comparable to that of the positive control under the experimental conditions. The observed healing responses appear to correlate with the phytochemical profile of the fractions, particularly their phenolic content. However, no direct mechanistic pathways or specific bioactive compounds were identified in this study. Therefore, the present findings provide preliminary *in vivo* evidence supporting

fraction-dependent biological activity and serve as a basis for further phytochemical characterization and mechanistic investigations.

Author Contributions

Conceptualization, M.R.E.; methodology, M.R.E., M.R.S., and D.R.G.; supervision, M.R.E. and D.R.G.; resources, M.R.E., I.F.Y., and M.S.R.; investigation, M.R.E., D.A.R., I.F.Y., and M.S.R.; formal analysis, I.F.Y., M.R.E., and M.S.R.; writing—original draft preparation, D.R.G., I.F.Y., M.S.R., and M.R.E.; writing—review and editing, D.R.G., M.S.R., and M.R.E. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

The animal study protocol was approved by the Research Ethics Committee of FKIK Universitas Jambi (Approval No. 986/UN21.8/PT.01.04/2024). All procedures complied with institutional guidelines.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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